REVIEW

Molecular pathology and genetics of pancreatic endocrine tumours

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Abstract

Pancreatic neuroendocrine tumours (PETs) are the second most frequent pancreatic neoplasms. Their poor chemosensitivity, high rate of metastatic disease and relatively long survival make PETs an ideal field to be explored for novel therapies based on specific molecular changes. PETs are generally sporadic but can also arise within hereditary syndromes, such as multiple endocrine neoplasia type 1, von Hippel–Lindau, neurofibromatosis type 1 and tuberous sclerosis complex, which represent a model for sporadic cases too. Among allelic imbalances, main genomic changes involve gain of 17q, 7q and 20q and loss of 11q, 6q and 11p, which identify regions of putative candidate oncogenes or tumour suppressor genes (TSGs), respectively, sometime with potential prognostic significance. Overexpression of Src-like kinases and cyclin D1 (*CCND1*) oncogene has been described. As for TSGs, P53 (TP53), DPC4/SMAD4 and *RB* (*RB1*) are not implicated in PET tumorigenesis, while for *p16INK4a* (*CDKN2A*), *TIMP3*, *RASSF1A* and *hMLH1*, more data are available, suggesting a role for methylation as a silencing mechanism. In the last decade, gene expression profile studies, analysis of microRNAs and, more recently, large-scale mutational analysis have highlighted commonly altered molecular pathways in the pathology of PETs. The roles of the mammalian target of rapamycin pathway, and its connection with Src kinases, and the activity of a number of tyrosine kinase receptors seem to be pivotal, as confirmed by the results of recent clinical trials with targeted agents. Mutations of *DAXX* and *ATRX* are common and related to altered telomeres but not to prognosis.

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Introduction

Pancreatic neuroendocrine tumours (PETs) represent only ~1% of all pancreatic neoplasms by incidence, but ~10% by prevalence, reflecting their relatively ‘indolent’ clinical course (Fitzgerald *et al.* 2008). However, the prognosis of PETs can be extremely heterogeneous, mainly depending on their staging and grading according to well-defined criteria (Panzuto *et al.* 2011). The clinical management of PETs is also challenging, as many present with metastatic disease at diagnosis and are not amenable for surgery, and few medical treatments have been proved effective (Walter *et al.* 2012).

In this view, the development of different novel agents aimed at targeting specific molecules seems a very attractive strategy, and while the approval of ‘targeted’ therapies (everolimus and sunitinib) have

raised enthusiasm in clinicians dealing with PETs (Jensen & Delle Fave 2011), a deeper knowledge of the molecular pathology is mandatory to implement such an approach. This paper summarises existing evidences about the molecular pathogenesis of PETs, presenting data from familial syndromes and genetic instability studies, as well as those examining the role of oncogenes, tumour suppressor genes (TSGs) and ‘targetable’ genes, including an insight into genome-wide studies.

Inherited PETs

A quote of PETs arises in the context of genetic syndromes, which also represent a reference model for the study of sporadic cases. The hereditary syndromes associated with PETs are listed in Table 1.

Table 1 Genetic syndromes associated with inherited pancreatic endocrine tumours, including clinical features and molecular defects

Syndrome	Gene	Gene function main molecular consequences	Major clinicals features	Patients with PET (%)	PET subtype	Metastatic PET (%)
Multiple endocrine neoplasia type I	Menin (11q13)	Oncosuppressor Deregulation of JunD, SMAD3 p27 ^{KIPI} p18 ^{Ink4c}	Two or more between: a) GEP-NET b) Parathyroid adenomas c) Pituitary adenoma	20–100%	100% NF 54% Gastrinomas 15% Insulinomas 3% Glucagonomas 1% GFRomas or VIPomas	< 10%
von Hippel– Lindau disease	VHL (3p25–26)	Oncosuppressor Overexpression of HIF and VEGF	One or two between: a) Retinal or cerebellar hemangioblastomas b) Renal cell carcinoma c) Pheochromocytoma	5–17%	80–100% NF	< 10%
von Recklinghausen's disease	NF1 (17q11.2)	Oncosuppressor Deregulation of Ras pathway (mTOR)	a) Café-au-lait skin spots b) Neurofibromas of any type and localisation	Rare	Duodenal somatos- tinomas (1–10%) Insulinomas (<1%)	–
Tuberous sclerosis complex	TSC1 (9q34) TSC2 (16p13.3)	Oncosuppressor Deregulation of mTOR pathway	a) Skin alterations b) Renal angiomyolipomas c) Mutiple and diffuse hamartomas d) Neurological alterations	Very rare	Mainly NF	–

GEP-NET, gastroenteropancreatic neuroendocrine tumour; PET, pancreatic neuroendocrine tumour; NF, non-functioning.

Multiple endocrine neoplasia type 1

The most frequent inherited syndrome associated with the occurrence of PETs is multiple endocrine neoplasia type 1 (MEN1), an autosomal dominant disorder (incidence 1:20 000–40 000) clinically defined by the presence of two or more of gastroenteropancreatic neuroendocrine tumours, parathyroid gland adenoma and hyperplasia and pituitary adenomas, with other neoplastic lesions occurring occasionally (Lemos & Thakker 2008). About 10% of PETs occur as a part of the MEN1 syndrome, which is the result of an inactivating mutation of a TSG located on chromosome 11q13 (Metz & Jensen 2008). Ten exons constitute the *MEN1* gene, and the corresponding protein, named MENIN, is involved in different functions, such as inactivation of transcription factors at a nuclear level (*JUND* and *SMAD3*), modulation of cell cycle inhibitors (enhancing p27^{KIPI} and p18^{INK4c} function) and interaction with DNA repair machinery. The final result is a negative control of the cell cycle (Karnik *et al.* 2005). The recent description of the crystal structure of MENIN allowed an improved description of its interaction with *JUND* and *MLL* (Huang *et al.* 2012). The mutations spectrum of *MEN1* is wide: about 1300 different germline mutations have been described and 10–12% of them occurred even in the absence of a family history. How mutations lead to cancer is not well understood, and the exact role of MENIN as a tumour suppressor is controversial (Jensen *et al.* 2008).

Gene mapping in MEN1 patients have shown loss of heterozygosity (LOH) in 50% of cases and that MEN1 carcinogenesis is sustained by a typical Knudson's two-hit mechanism. LOH of *MEN1* and other somatic mutations on the wild-type allele work as a second hit after a first germline mutation. PET patients with MEN1 are clinically similar to sporadic cases except for their earlier age at disease presentation. Neoplasms are non-functioning (NF)-PETs in about 80% of cases, gastrinomas in 54%, insulinomas in 15–20%, glucagonoma in 3% and rarely VIPomas or GRFomas. NF-PETs are found microscopically in 80–100% of cases, <15% being symptomatic (Jensen *et al.* 2008). Although 20–60% of MEN1 patients have Zollinger–Ellison syndrome, gastrinomas arise far more frequently in the duodenum than in the pancreas (Metz & Jensen 2008). Apart from the 10% associated with the typical MEN1 syndrome, a high percentage of sporadic PETs present with molecular abnormalities of the *MEN1* gene or of its function, thus suggesting its crucial role in their pathogenesis. Mutation of *MEN1* and allelic loss of chromosome 11q are the most common genetic alterations, especially among NF-PETs (Moore *et al.* 2001a, Perren *et al.* 2007). Mutations of *MEN1* have been found in 30% of sporadic NF-PETs, 7% of insulinomas, 36% of gastrinomas, 67% of glucagonomas and 44% of VIPomas (Moore *et al.* 2001a). Recent data from Jiao *et al.* (2011) report 44% of inactivating mutations in *MEN1* in a series of sporadic PETs.

Moreover, losses at 11q13 and/or more distal parts of the long arm of chromosome 11 are also relatively frequent events (38.6% of NF-PETs and about 15–20% of gastrinomas and insulinomas). Thus, haploinsufficiency of the *MEN1* could represent a sufficient initiating factor for PET development (Görtz *et al.* 1999, Goebel *et al.* 2000). In another series of 169 sporadic PETs, *MEN1* mutational status and immunohistochemical and western blot analysis were performed with about 25% of sporadic PETs harbouring a somatic *MEN1* mutation, scattered throughout the coding sequence and splice sites (Corbo *et al.* 2010). Further studies are needed to better understand the complex MENIN pathway and its interactors involved at different levels in cell cycle regulation.

von Hippel–Lindau disease

PETs also occur in von Hippel–Lindau (VHL) disease (incidence around 1:50 000), an autosomal dominant phakomatosis. VHL disease diagnosis is made by identification of at least one of pheochromocytoma, renal cell carcinoma, retinal or cerebellar hemangioblastoma and other multiorgan neoplasms occurring less frequently, such as pancreatic cysts and PETs (Corcos *et al.* 2008).

The *VHL* gene is an oncosuppressor composed of three exons and located on 3p25–26. It encodes, by alternative splicing, two proteins (pVHL), respectively, of 213 and 160 aminoacids, involved in assembling the ubiquitin complex, which in normoxia binds and inactivates hypoxia-inducible factor 1 α (HIF1 α ; Jensen *et al.* 2008).

Inactivating mutations of the *VHL* gene result in HIF dysregulation, with subsequent overexpression linked to the hypoxia-driven angiogenic pathways. As recently highlighted in non-tumoral pancreas of VHL patients examined for microadenomatosis, overexpression of the pVHL/HIF pathway proteins was immunohistochemically demonstrated as an early molecular event occurring before PET development (Périgny *et al.* 2009).

More than 300 *VHL* germline mutations have been described with different phenotypical expression. VHL is typically characterised by the presence of multiple benign pancreatic cysts (occurring in 50–75% of cases) while PETs occur in a lower proportion of cases (5–17%; Mukhopadhyay *et al.* 2002). LOH in the *VHL* gene or, less frequently, promoter methylation or *de novo* mutation are the most common alterations detected in VHL-associated PETs (Lott *et al.* 2002).

VHL-associated PETs are often small (measuring <2–3 cm), multiple, non-functional tumours and in only rare cases (<10–20%) can develop liver metastases. Compared with sporadic PETs, their prognosis is usually better. *VHL* mutations rarely occur in sporadic PETs. However, in a recent study in 35 sporadic PETs, *VHL* gene inactivation either by promoter methylation or by deletion

was associated with active hypoxia signalling and shortened disease-free survival (Schmitt *et al.* 2009).

von Recklinghausen's disease or neurofibromatosis type 1

Neurofibromatosis type 1 (NF1) is an autosomal dominant phakomatosis with an incidence of 1:3000 and a high penetrance, defined by multiple café-au-lait skin spots and ubiquitous neurofibromas. Another typical feature is the high probability (3–30%) of developing different types of cancer such as gliomas, myeloid leukaemia and pheochromocytoma (McClatchey 2007). PETs are detected more rarely in NF1 than in MEN1 or VHL disease.

The mutation of the *NF1* gene (17q11.2) is responsible for NF1 disease. This gene encodes for neurofibromin, a GTPase protein involved as a negative regulator of the Ras pathway, and in particular of mammalian target of rapamycin (mTOR) function, whose overactivation may be a key event in PET development in such cases (Rosner *et al.* 2008a). Different *NF1* mutations have been described (about 50% arising *de novo*) with possible genotype/phenotype associations identified (McClatchey 2007).

Tuberous sclerosis complex

Among the inherited diseases, tuberous sclerosis complex (TSC) is the least frequently associated with PETs, another hereditary autosomal dominant disease with an incidence of 1:10 000. Clinical presentation includes typical skin lesions (hypomelanotic macules, facial angiofibromas, ungual fibromas, Shagreen's patches and forehead plaque), renal angiomyolipomas, hamartomas, mental retardation and neurological disorders. PETs are very rarely diagnosed within TSC (Curatolo *et al.* 2008). *TSC1* (9q34) and *TSC2* (16p13.3), respectively, encoding for hamartin and tuberin, are responsible for this disease. These two proteins together control cell proliferation, through interaction with PI3-kinase–mTOR pathway activity and insulin receptor signalling.

PETs associated with TSC are mainly NF, with clinical characteristics comparable to sporadic cases (Rosner *et al.* 2008b). *TSC2* mutations have been found in 8.8% of sporadic PETs throughout exomic sequencing (Jiao *et al.* 2011).

Genetic instability in sporadic pancreatic endocrine tumours

Genome-wide studies

Alterations in DNA copy number are common events occurring during tumour development that may be

revealed by different methods including karyotyping, comparative genomic hybridisation (CGH), fluorescence *in situ* hybridisation, microsatellite analysis or single nucleotide polymorphism allelotyping.

Most of the available data about CGH study in PETs refer to small heterogeneous series. Moreover, different tumour classifications have been used by investigators making the possible analysis of the different PET subtypes difficult. Our report aims at organising data, separating non-functional (101 tumour samples) from functional PETs and, among these, taking into account possible differences between benign (116) or malignant insulinomas (30) and gastrinomas (31) (Chung *et al.* 1998, Terris *et al.* 1998, Speel *et al.* 1999, 2001, Stumpf *et al.* 2000, Rigaud *et al.* 2001, Tonnies *et al.* 2001, Zhao *et al.* 2001, Florida *et al.* 2005, Jonkers *et al.* 2005, Nagano *et al.* 2007).

The most frequent findings about losses or gains are listed in Tables 2 and 3 respectively. Candidate TSGs and oncogenes, the associated disorders for which a pathogenic link has been already described, and, finally, their possible prognostic relevance are also described. As for the 31 gastrinomas investigated, loss of 3p (19%) and gain of 9p (29%) represented the most common chromosomal aberrations.

In benign insulinomas, most frequent losses were found on 11q (19%), Xq (18%) and 1p (17%) while most frequent gains regarded 9q (41%), 7p (20%) and 7q e 5q (both 19%). Malignant insulinomas harboured more genomic alterations than benign ones. In particular, most frequent losses were found on 6q (70%), Y (43%) and 2q (33%), while main gains involved 17q (57%), 17p (53%) and 12q (53%).

On the whole, NF-PETs seem to present the highest rate of genomic aberrations, followed by malignant insulinomas, with benign insulinomas and gastrinomas presenting the lowest amount of changes. This tendency is consistent with the finding that PETs larger than 2 cm exhibited significantly more aberrations than lesions smaller than 2 cm (Speel *et al.* 1999, Zhao *et al.* 2001).

Loss of heterozygosity

As shown for other tumour types, malignant progression of PETs is driven by progressive accumulation of multiple genetic changes (Speel *et al.* 1999, Zhao *et al.* 2001, Jonkers *et al.* 2005), and accumulating evidence suggests that PETs from patients with advanced disease harbour more genetic aberrations than tumours from patients with localised disease. Several LOH studies using microsatellite markers demonstrated that LOH at long arm of chromosome 1 (Ebrahimi *et al.* 1999, Guo *et al.* 2002a, Chen *et al.* 2003, Yang *et al.* 2005), at short arm of chromosome 3 (Nikiforova *et al.* 1999, Barghorn *et al.* 2001, Guo *et al.* 2002b, Amato *et al.* 2011)

and at long arm of chromosome 22 (Wild *et al.* 2001, 2002) is a common event among different PET subtypes and is significantly associated with the presence of hepatic metastases regardless of tumour type (Table 2).

LOH on chromosome 17p13 was detected in about 24% of the chromosomal loci scanned in a serie of 20 PETs, with the number of allelic losses significantly correlated with the biological features and malignancy of the tumours (Beghelli *et al.* 1998). The absence of *P53* (*TP53*) gene mutations in most of these tumours suggests the existence of another TSG in the same chromosomal area.

In malignant insulinomas, the gain of 17q is frequently found (more than 50% of cases). This chromosomal alteration may suggest a role for *HER2/NEU* (*ERBB2*; located on chromosome 17q21), whose overexpression is a well-known negative prognostic marker in breast and gastroesophageal tumours. *HER2/NEU* gene amplifications were identified in 5 of 11 gastrinomas and often associated with advanced or metastatic disease (Evers *et al.* 1994). In another study, the amplification of *HER2/NEU* was described in 14% of gastrinomas, with a relationship between its mRNA levels in tumour cells and the presence of liver metastases (Goebel *et al.* 2002).

Interesting data also come from sex chromosomes. Xq loss was found in 20% of insulinomas (see references in genome-wide study paragraph). An association between Xq loss and advanced disease was also described, with a possible pathogenetic role of X chromosome changes in influencing the malignant behaviour of neuroendocrine tumours (Speel *et al.* 1999).

To further support this hypothesis, only 4.5% of benign tumours (gastric and pancreatic) harboured LOH on chromosome X compared with 60% found in malignant tumours (Pizzi *et al.* 2002).

In a series of specimens from 16 female patients with gastrinomas, 56% presented with X chromosome LOH, which was significantly associated with aggressive tumour growth as well as with large primary tumour size and pancreatic primary tumour origin (Chen *et al.* 2004). In another study, loss of chromosome X was described in 40% of cases of PETs from females, while loss of chromosome Y was present in 36% of cases of PETs from males. In the same study, sex chromosome alterations were associated with the presence of metastases, higher proliferation index and worse prognosis (Missiaglia *et al.* 2002).

Furthermore, the presence of allelic loss on the X chromosome was more frequent in poorly differentiated neuroendocrine (in 72% of primitive tumours and in 81% of metastases) than in well-differentiated neuroendocrine tumours (in 55% of primitive tumours and in 48% of metastases; Azzoni *et al.* 2006).

Array-CGH technology can improve the resolution of conventional CGH. This technique has recently

Table 2 Main losses in sporadic PETs and possible related TSGs

Location	Prognostic relevance	Frequency of losses among PET subtypes (%)				Putative TSGs	Associated disorder
		NF	B Ins	M Ins	Gas		
11q		39	19	23	13	<i>MEN1</i> <i>PLCB3</i> <i>SDHD</i> <i>TSG11</i> <i>HHPT</i> <i>BRCC2 (BRCA2)</i> <i>ATM</i>	MEN1 syndrome Intestinal carcinoids, paraganglioma and pheochromocytoma Non-small cell lung cancer Hereditary hyperparathyroid – Jaw tumour syndrome Breast cancer Ataxia telangiectasia and breast cancer Melanoma
6q	Associated with liver metastases (Speel <i>et al.</i> 1999, Barghorn <i>et al.</i> 2001)	38	3	70	0	<i>AIM1</i> <i>CCNC</i> <i>PTPRK</i> <i>LOT1 (PLAGL1)</i> <i>CX43 (GJA1)</i>	Transient neonatal diabetes mellitus Oculodentodigital dysplasia, hypoplastic left heart syndrome and atrioventricular septal defect Wilms tumour type 1, Denys–Drash syndrome, WAGR syndrome, Frasier syndrome and isolated diffuse Mesangial sclerosis von Hippel–Lindau syndrome and renal cell carcinoma Colorectal cancer and HNPCC
11p		34	15	23	3	<i>WT1</i>	Wilms tumour type 1, Denys–Drash syndrome, WAGR syndrome, Frasier syndrome and isolated diffuse Mesangial sclerosis
3p	Associated with liver metastases (Ebrahimi <i>et al.</i> 1999, Speel <i>et al.</i> 1999)	27	0	20	19	<i>VHL</i> <i>hMLH1</i> <i>RARβ</i> <i>β-Catenin</i> <i>RASSF1A</i>	von Hippel–Lindau syndrome and renal cell carcinoma Colorectal cancer and HNPCC Digestive endocrine tumours Lung cancer
1p	Associated with liver metastases (Ebrahimi <i>et al.</i> 1999)	28	17	27	3	<i>P73 (TP73)</i> <i>p18/INK4 (CDKN2A)</i> <i>RUNX3</i>	
10q		26	0	23	3	<i>MGMT</i>	Endometrial and follicular thyroid cancer, meningioma
1q	Associated with metastases and aggressive growth (Guo <i>et al.</i> 2002a,b, Chen <i>et al.</i> 2003 and Yang <i>et al.</i> 2005)	24	15	20	10	<i>PTEN</i> <i>HHPT2</i> <i>MDA7/IL24</i>	Hereditary hyperparathyroid – Jaw tumour syndrome Several cancer cell lines

NF, non-functioning; F, functioning; B Ins, benign insulinoma; M Ins, malignant insulinoma; Gas, gastrinoma; PETs, pancreatic endocrine tumours; TSGs, tumour suppressor genes.

been applied to PETs. In a series of 27 insulinomas, an array-based CGH analysis detected loss of chromosomes 11q and 22q and gain of chromosome 9q in >50% of cases; notably, those first two alterations were underestimated by conventional CGH analysis (Jonkers *et al.* 2006). In another array-based CGH study, nearly all tumours (98%) had chromosomal alterations and more alterations were described in metastases than in matched primary lesions (Hu *et al.* 2010).

Genetic alterations of oncogenes and TSGs

The role of K-RAS in PETs has been investigated by a number of authors, with findings suggesting limited

relevance if any, thus differentiating PETs from their exocrine counterpart (Evers *et al.* 1994, Pavelic *et al.* 1996, Moore *et al.* 2001b, Goebel *et al.* 2002). The RAF pathway has also been investigated and does not seem to be directly involved (Tannapfel *et al.* 2005). However, findings of the inactivation of the TSG Ras association domain family 1 (*RASSF1*; see below) may suggest distinct mechanisms by which the Ras pathway is activated in PETs.

Src is another proto-oncogene, which through its kinase activity transduces signals from the plasma membrane, conveyed by internal and external cues into various cellular responses, from cell cycle control, to cell adhesion and motility. Among the different members of the Src family kinases (SFKs), LCK has

Table 3 Main gains in sporadic endocrine tumour of the pancreas and possible related oncogenes

Location	Prognostic relevance	Gain (%)				Putative oncogenes	Associated disorder
		NF	B Ins	M Ins	Gas		
17q	Associated with malignant behaviour in tumours <2 cm (Speel <i>et al.</i> 2001)	41	5	57	12	NEU/ERB2	Breast cancer
7q		35·9	19	47	12	HGF	Gastric cancer and hepatocellular carcinoma
20q		30·7	12	43	6	C-MET STK15/BTAK (AURKA)	Breast cancer, ovarian and digestive carcinomas
9p		19·2	6	13	29	JAK2	Acute myelogenous leukaemia and myeloproliferative disorder
7p		28	20	37	6	Oncogene ovc RAGA (RRAGA) EGFR/ERBB1	Ovarian carcinoma
9q		26·9	41	43	12	CDK9 cABL	Bladder, breast, epidermoid carcinoma and glioblastoma
						NOTCH1	Breast cancer, head and neck squamous carcinoma
						LMX1B	Chronic myeloid leukaemia and rat insulinoma (cell lines)
							SCLC and T-cell acute lymphoblastic leukaemia

NF, non-functioning; B Ins, benign insulinoma; M Ins, malignant insulinoma; Gas, gastrinoma.

been found to be overexpressed at the RNA and protein levels in tissue from patients with advanced PETs in progressive disease (Capurso *et al.* 2006). The expression and activity of different SFKs have been reported in PET cell lines and samples, and the pharmacological inhibition of the activity of SFK has been found to decrease the capacity of PET cells to adhere, spread and migrate (Di Florio *et al.* 2007). Furthermore, a novel role for SFKs in controlling mTOR activity during adhesion has been reported. The concomitant inhibition of SFK and mTOR activities strongly impaired PET cell line growth, without triggering the activation of a survival response dependent on PI3K/AKT signalling (Di Florio *et al.* 2011).

Aberrant activation of the Wnt/ β -catenin signaling pathway is a hallmark of many tumours. Its role can be assessed either by studying mutations of the involved genes or by evaluating the accumulation of the β -catenin protein in the nucleus, which is considered a hallmark of Wnt activation. Neither β -catenin mutations nor its nuclear accumulation have been found in PETs in a first study (Hervieu *et al.* 2006), while some abnormalities have been reported by other authors (Chetty *et al.* 2008).

The few other oncogenes that seem to have a significant role in PETs are the antiapoptotic factor BCL2, which has been detected in about a half of samples, the cyclin D1 (CCND1), for which a correlation with the stage of the disease has been reported

(Chung *et al.* 2000, Guo *et al.* 2003), and the transcription factor c-MYC, whose expression has been ascertained by a number of investigators (Roncalli *et al.* 1991, Pavelic *et al.* 1996, Wang *et al.* 1997, Moore *et al.* 2001b).

As far as regards TSGs, the role of *MEN1* and *VHL* mutations, either in familial syndromes or in sporadic PETs, has been summarised in the previous paragraphs.

The role of the *P53* TSG has been widely investigated. A rationale for such investigations comes from studies in mice with *p53* mutations who develop PETs (Harvey *et al.* 1995). Nevertheless, most studies found no mutations of *P53* and/or no overexpression of the mutated protein in human PETs (Evers *et al.* 1994, Wang *et al.* 1995, Bartz *et al.* 1996, Pavelic *et al.* 1996, Moore *et al.* 2001b, Goebel *et al.* 2002). These data suggest that findings of LOH at 17q13 may be related to other unknown TSGs. However, a recent paper reported that, although *P53* mutations were rare in PETs, different key negative regulators of *P53* protein levels and activity, such as MDM2 (22%), MDM4 (30%) and WIP1 (51%), are overexpressed in PETs (Hu *et al.* 2010).

Similarly, although LOH at 18q is fairly frequent in PETs, the *DPC4/SMAD4* gene is rarely mutated (Pavelic *et al.* 1996, Bartsch *et al.* 1999, Moore *et al.* 2001b), and the retinoblastoma TSG (*RB*) gene is also not implicated (Chung *et al.* 1997). The p16 kinase inhibitor gene (*CDKN2A*), a TSG associated with familial melanoma,

has been found to be inactivated in PETs. The gene is inactivated, either at the genetic or epigenetic level, more frequently in patients with gastrinoma than in those with insulinoma or NF-PETs (Evers *et al.* 1994, Serrano *et al.* 2000, Moore *et al.* 2001b).

RASSF1 has properties compatible with a tumour suppressor function, because the protein contains a putative Ras association domain. RASSF1A is one of the most frequently inactivated proteins in solid cancers, either by mutations or by epigenetic inactivation; PETs do not make an exception, as hypermethylation was detected in most tumours in a small series (Dammann *et al.* 2003), while the deletion of the 3p21.3 area seemed associated with an aggressive behaviour (Pizzi *et al.* 2005).

Molecular alterations of possible 'targetable' genes

Alteration of the PI3K/protein kinase B/AKT/mTOR pathway

mTOR is a serine–threonine kinase that plays a key role in cell growth and proliferation. The PI3K/AKT/mTOR pathway is one of the most relevant transduction pathways, and mTOR phosphorylates downstream targets (S6K and 4E-BP1) controlling mRNA translation. Different players are involved in the balance of mTOR activity, including TSGs and oncogenes. Among them are the TSGs PTEN, NF1, LKB1 (STK11) and TSC1/TSC2, which have all been reported to have decreased activity in PETs (Averous & Proud 2006).

A number of studies investigated the expression of genes belonging to the PI3K/AKT/mTOR pathway in PETs, suggesting its activation. Immunohistochemical expression of p-mTOR has been found in four of ten patients with PETs, while pAKT expression was infrequent (Shida *et al.* 2010). Kasajima *et al.* (2011) demonstrated that expression and activity of mTOR were higher in foregut (gastric, duodenal and pancreatic) than in midgut tumours, correlating with distant metastases. The activity of the mTOR pathway, as assessed by phosphorylation of 4E-BP1, has also been reported to be an independent factor of poor prognosis (Di Florio *et al.* 2011).

The mechanisms leading to overactivation of the mTOR pathway in PETs, however, are not completely understood. Interestingly, *in vivo* and *in vitro* models show that AKT function is down-regulated by MENIN. In fact, MENIN suppresses AKT-induced proliferation and anti-apoptosis by reducing translocation of AKT from the cytoplasm to the plasma membrane during growth factor stimulation, suggesting an important novel role for MENIN as a negative regulator of AKT kinase activity (Wang *et al.* 2011). On the other hand,

although losses at 16p, where *TSC2* is located, and at 10q where *PTEN* is encoded, are rather frequent findings in PETs, mutations of these genes, or of other genes of the mTOR pathway, are not very common events (Perren *et al.* 2000, Rigaud *et al.* 2001, Corbo *et al.* 2012, Jiao *et al.* 2011). However, the activity of PTEN and TSC2 may be impaired by other mechanisms, or by differential subcellular localisation, and their reduced expression at the protein level is significantly associated with shorter overall and disease-free survival (Missiaglia *et al.* 2010). The possible role of the Src pathway in controlling mTOR activity in PET cells has been discussed earlier (Di Florio *et al.* 2011).

Further molecular pathology studies should be focused on the mechanisms controlling the mTOR pathway in PETs, with the aim to eventually select which patients would benefit from treatments targeting these molecules or in view of possible treatment with drugs targeting genes upstream of mTOR.

Alteration of growth factors and receptors

The expression of growth factors and their receptors offers the opportunity for targeted therapy. The surface of PET cells presents several growth factor receptors, including receptor tyrosine kinases such as epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF1R), hepatocyte growth factor (HGF), the stem cell factor (SCF) receptor c-KIT and the platelet-derived growth factor receptors (PDGFRs; Srivastava *et al.* 2001, Peghini *et al.* 2002, Fjallskog *et al.* 2003, Furukawa *et al.* 2005, Corbo *et al.* 2012).

EGFR is a member of the erbB/human EGFR family of tyrosine kinases, which is activated by interaction with the cognate ligand. The activity of the receptor as assessed by its phosphorylation has been described as a factor associated with negative outcome in PETs (Papouchado *et al.* 2005), although the role of the EGFR seems more relevant in intestinal neuroendocrine tumours. In gastrinomas (Peghini *et al.* 2002), EGFR was overexpressed in 18% of cases and correlated with the presence of metastases and lower curability. IGF1 and IGF1R are also frequently expressed in gastrinomas, and IGF1R levels are correlated with tumour growth, aggressiveness and development of metastases (Furukawa *et al.* 2005).

c-KIT (CD117) is a type III tyrosine kinase receptor that, once activated by its ligand, SCF, induces dimerisation and autophosphorylation of the receptor at specific tyrosine regions, resulting in intracellular signal transduction. Abnormal expression of c-KIT and/or SCF has been described in a variety of solid tumours. Several studies have investigated the expression of c-KIT in PETs by immunohistochemistry. While earlier papers showed inconsistent results (Fjallskog *et al.* 2003, Koch *et al.* 2006), in more recent

studies, a prognostic role for c-KIT has been highlighted. Zhang *et al.* (2009) reported that immunostaining for c-KIT is a significant independent factor associated with poor prognosis in a series of 97 PETs. Corbo *et al.* (2012) analysed a series of 140 PETs and found no mutations of *EGFR*, *HER2* and *PDGFR α* (*PDGFRA*), while *KIT* was mutated in only one case. However, at the protein level, c-KIT membrane immunostaining was significantly associated with tumour aggressiveness and shorter patient survival. These results could be of clinical interest, as c-KIT is one of the different tyrosine kinases, together with VEGFR, PDGFR, FLT3 and RET, targeted by sunitinib, which has been approved for use in PETs.

The VEGF pathway is also of special interest in PETs, as angiogenesis switch, coupled by progressive expression of VEGF and its receptors, are key mechanisms in the transgenic mouse model (Rip1-Tag2) in which the animals develop hyperplastic islets and PETs (Hanahan & Folkman 1996). Data obtained from PET patients are somehow more controversial, with some studies reporting that expression of VEGF correlates with a more aggressive tumour behaviour (Rubbia-Brandt *et al.* 2004, Zhang *et al.* 2007) and others that malignant tumours show lower VEGF expression than benign ones (Couvelard *et al.* 2005). More studies are needed to specifically evaluate the clinical relevance of the expression of all these tyrosine kinases as possible prognostic markers, or as therapeutic targets, especially in patients treated with their inhibitors.

Microarray studies and gene expression profiling

In the past decade, the advent of microarray technology, which allows the investigation of levels of expression of thousands of genes at the same time, has been widely applied to different cancer types including PETs.

While this approach has a number of potential advantages and may prove useful to investigate markers and targets of potential interest for treatment or prediction of prognosis, its clinical use is often hampered by a number of limitations, which include heterogeneity of patients, samples and microarray platforms. Moreover, the significance of gene expression profile studies is limited in the absence of an appropriate statistical analysis and independent validation.

Microarray analysis of expression profiles has also been used to investigate PETs, with a number of different strategies. These studies are summarised in Table 4 (Maitra *et al.* 2003, Bloomston *et al.* 2004, Hansel *et al.* 2004, Dilley *et al.* 2005, Capurso *et al.* 2006, Couvelard *et al.* 2006, Duerr *et al.* 2008, Missiaglia *et al.*

2010). The different samples and methods account for the expected poor reproducibility of the results. Indeed, while a part of these studies investigated the expression profile of PET samples compared with a normal reference (either purified pancreatic islets and/or other normal pancreatic samples), others compared PETs with different clinical behaviour (metastatic vs non-metastatic or NF vs functioning). In some instances, a comparison between primary pancreatic lesions and their distant metastases was also presented, with interesting findings of a striking similarity in the expression profile of matched lesions (Capurso *et al.* 2006, Missiaglia *et al.* 2010).

Some interesting candidates for further evaluation as prognostic factors or therapeutic targets emerged. In particular, the study by Missiaglia *et al.*, which investigated by far the largest samples set, identified alteration of the mTOR pathway (see earlier), and the study by Capurso *et al.* highlighted the overexpression of SFKs in PETs.

MicroRNAs are small non-coding RNAs that can control the expression of specific mRNAs through their degradation or translation inhibition. The global expression of microRNAs in PETs has been only investigated by Roldo *et al.* (2006), with a specific custom microarray using a large set of PETs, compared with normal pancreas. The authors could demonstrate that a specific pattern of microRNA distinguishes PETs. Some of these microRNAs were identified and associated with clinical features, such as miR-204, primarily expressed in insulinomas, or with prognostic factors, such as miR-21, which was strongly associated with both high Ki67 and liver metastases (Roldo *et al.* 2006). Interestingly, higher expression of miR-21 was also linked with lower expression of PTEN, further supporting the role of the PI3K/AKT/mTOR pathway.

Summary and conclusion

This paper extensively summarises the existing data about molecular and genetic alterations in PETs, including PETs arising within genetic syndromes, which represent a reference model for the study of sporadic cases. A critical review of the large amount of genome-wide studies and of those analysing genetic alterations of oncogenes and TSGs has been performed, along with an insight into microarray studies. Although all these different methods are hardly comparable, some considerations and intriguing findings deserve to be highlighted.

The molecular pathogenesis of PETs seems largely different from that of pancreatic adenocarcinoma (Fig. 1), confirming PETs as a distinct entity with a peculiar molecular background.

Table 4 Summary of gene expression profile studies on pancreatic endocrine tumours

Reference	Samples	Comparison(s)	Platform	Upregulated genes	Downregulated genes	Relevant genes	Confirmation
Capurso <i>et al.</i> (2006)	13 NF-PETs (eight primary, five metastasis), three cell lines (BON CM QGP) and four purified islets	1. PETs vs islets 2. Primary vs metastases	Affymetrix U133A + B	668 –	323 –	<i>LOK</i> , <i>BIN1</i> , <i>BST2</i> and <i>SERPINA10</i>	IHC qRT-PCR
Maitra <i>et al.</i> (2003)	Eight NF-PETs (no distant metastases) and three purified islets	PETs vs islets	Affymetrix U133A	66	119	<i>IGFBP3</i> , p21 fibronectin and <i>MIC2</i> (CD99)	IHC
Dilley <i>et al.</i> (2005)	Eight PET samples from six MEN1 patients (two insulinomas, two NF, one vipoma and one gastrinoma)	PETs vs islets	Affymetrix U95AV2	45	148	<i>IER3</i> , <i>IAPP</i> , <i>SST</i> and <i>PHLDA2</i>	qRT-PCR
Bloomston <i>et al.</i> (2004)	Pooled biopsies from nine PETs, normal pancreas, PDAC and CP	PETs vs normal pancreas	Affymetrix U133A	NS	NS	<i>ANG2</i> (<i>ANGPT2</i>), <i>NPDC1</i> , <i>ELOVL4</i> and <i>CALCR</i>	IHC RT-PCR
Duerr <i>et al.</i> (2008)	24 PETs (nine insulinomas, four NF, three gastrinoma, one glucagonoma, one ACTHoma and one PTHRPoma) and six GI carcinoids	1. 12 WDET's vs 7 WDECs 2. PETs vs carcinoids	Affymetrix U133A	71 228	41 157	<i>FEV</i> , <i>NR4A2</i> , <i>ADCY2</i> and <i>GADD45β</i> (<i>GADD45B</i>)	qRT-PCR
Hansel <i>et al.</i> (2004)	12 primary PETs	Seven metastatic vs five non-metastatic	Affymetrix U133A + B	65	57	<i>IGFB3</i> and <i>MET</i>	IHC
Couvelard <i>et al.</i> (2006)	24 well-differentiated PETs (20 NF)	12 WDET's vs 12 WDECs	Sanger Centre Custom 10k	72	51	<i>MDR1</i> (<i>ABCB1</i>), <i>MKK4</i> (<i>MAP2K4</i>) <i>E-selectin</i> and <i>CD34</i>	IHC
Missiaglia <i>et al.</i> (2010)	72 primary PETs (56 NF, 15 insulinomas, one gastrinoma), seven matched metastases, five normal pancreas and five purified islets	1. NF-PETs vs normal 2. Insulinoma vs normal 3. NF WDET vs insulinoma	18-5 K oligoarray (Ohio State University Cancer Center)	189 113 161	55 25 101	<i>TSC2</i> , <i>PTEN</i> , <i>SSTR2</i> and <i>FGF13</i>	IHC (TMA) qRT-PCR

TSG, tumour suppressor gene; WDET, well-differentiated endocrine tumour; WDEC, well-differentiated endocrine carcinoma; NF, non-functioning; NS, not specified; IHC, immunohistochemistry.

Indeed, the most typical and frequent molecular abnormalities occur in the *MEN1* gene, whose role needs further investigation, especially regarding the mechanisms leading to alteration of protein distribution in sporadic PETs. The dysregulation of the PI3K/AKT/mTOR signalling pathway at some level, due to mutations, loss or decreased activity of the TSGs PTEN or TSC, or to overexpression of upstream genes, appears to be another typical molecular signature in PETs, which has a prognostic relevance.

These findings are in line with the results of a recent large-scale mutational analysis reported by Jiao *et al.* (2011), where somatic mutations in *MEN1* and mTOR pathway genes emerged as the most frequent molecular events in PETs. In this study, the most frequent mutations (43% of PETs) were in the *DAXX* or *ATRX* genes; *DAXX* is a histone chaperone and the *ATRX*–*DAXX* complex assembles histones. These mutations affect telomere lengthening and genome stability but do not seem related with patient prognosis.

Some of these reported molecular alterations may prove important as a rationale for therapies with targeted agents. Two large phase 3, multicentre, double-blind, randomised, placebo-controlled trials testing the mTOR inhibitor everolimus (Afinitor, Novartis) and the multi-tyrosine kinase inhibitor sunitinib (Sutent, Pfizer) in malignant PETs were recently published, showing positive significant results

in terms of tumour response and overall survival (sunitinib) and progression-free survival (everolimus) (Raymond *et al.* 2011, Yao *et al.* 2011); both these drugs have been approved by the US Food and Drug Administration for the treatment of progressive, unresectable, locally advanced or metastatic PETs. However, molecular or surrogate markers able to predict the response of PET patients to treatment with either Afinitor or Sutent are not available, and the capacity of PET cells to develop escape pathways that evoke pro-survival feedback responses and the existence of crosstalk between different molecular pathways in this cancer type have been poorly investigated. In this view, a role for the Src pathway in PET cells deserves further investigation. Interestingly, a very recent paper underlined a relevant role for Src in stem cells isolated from intestinal carcinoid cells, further suggesting that this pathway may prove specifically relevant for neuroendocrine tumours (Gaur *et al.* 2011).

It is likely that, in the next decade, a more extensive application of new (or 'next generation') DNA sequencing technologies (Ding *et al.* 2010) will be able to characterise the individual patient's disease and to determine potential treatment modalities in PETs too. In this future scenario, sequencing of many tumours will allow an evaluation beyond individual genes to the consideration of pathways that may be 'druggable' in PETs.

Figure 1 Schematic of main molecular alterations described in PETs. A number of growth factors (IGF, EGF, SCF, PDGF and VEGF) and their receptors are active in PET cells. Among the transduction pathways, the PI3K/AKT/mTOR one seems particularly relevant. The activity of the mTOR pathway is negatively controlled by PTEN, the TSC complex, NF1 and MENIN whose expression or function is reduced in PET cells. Higher expression of miR-21 is also associated with lower PTEN levels. Src also controls mTOR activity. Mutations of *DAXX* and *ATRX* are common events and are related with abnormal telomeres.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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